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(57) Abstract

A method is provided for identifying, isolating, and producing lipooligosaccharide (LOS) mutants of gram–negative bacterial pathogens. The method comprises mutating the laft gene of a gram–negative bacterial pathogen so that there is a lack of a functional Lipid A fatty acid transferase protein. The resulting LOS mutants lack one or more secondary acyl chains as compared to the LOS contained in the wild type gram–negative bacterial pathogen. The LOS isolated from the laft mutants displays substantially reduced toxicity as compared to that of the wild type strain. Also, the present invention provides methods for using a vaccine formulation containing the laft mutants, the endotoxin isolated therefrom, or the endotoxin isolated therefrom which is then conjugated to a carrier protein, to immunize an individual against infections caused by gram–negative bacterial pathogens by administering a prophylactically effective amount of the vaccine formulation.

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LAFT MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA

Field of the Invention

The present invention relates to compositions comprising altered endotoxin lipooligosaccharide (LOS) of gram-negative bacterial pathogens. More particularly, the present invention relates to the making of a form of endotoxin, by a genetically engineered gram-negative pathogen, which lacks a substantially toxic Lipid A portion of LOS. Also disclosed are prophylactic and therapeutic uses of the substantially detoxified endotoxin, and of mutant gram-negative bacteria producing the substantially detoxified endotoxin.

Background of the Invention

Gram-negative bacteria have an outer membrane comprised of components including proteins, lipoproteins, phospholipids, and glycolipids. The glycolipids comprise primarily endotoxin lipopolysaccharides (LPS) or lipooligosaccharides (LOS), depending on the genus of bacteria. LPS are molecules comprised of a) a Lipid A portion which consists of a glucosamine disaccharide that is substituted with phosphate groups and long chain fatty acids in ester and amide linkages; b) a core polysaccharide which is attached to Lipid A by an eight carbon sugar, KDO (ketodeoxyoctonoate), and heptose, glucose, galactose, and N-acetylglucosamine; and c) an 0-specific side chain comprised of repeating oligosaccharide units which, depending on the genera and species of bacteria, may contain mannose, galactose, D-glucose, N-acetylgalactosamine, N-acetylglucosamine, L-rhamnose, and a dideoxyhexose (abequose, colitose, tyvelose, paratose, trehalose). while LOS has a structure similar to LPS in that LOS also contains a Lipid A portion and a complex carbohydrate structure, there are differences, including the lack of repeating 0-side chains in LOS.

The major antigenic determinants of gram-negative bacteria are believed to reside in the complex carbohydrate structure of LOS. These carbohydrate structures may vary for different species of the same genus of gram-negative bacteria by varying one or more of the sugar composition; the sequence of

oligosaccharides; the linkage between the oligosaccharides; and substitutions/modifications of an oligosaccharide (particularly a terminal oligosaccharide).

LOS has been considered as a bacterial component which has potential as vaccine immunogens because of the antigenic determinants ("epitopes") residing in its carbohydrate structures. However, the chemical nature of LOS prevents its use in vaccine formulations; i.e., active immunization with LOS is unacceptable due to the inherent toxicity of the Lipid A portion. The pathophysiologic effects induced (directly or indirectly) by Lipid A of LOS in the bloodstream include fever; leucopenia; leucocytosis; the Shwartzman reaction; disseminated intravascular coagulation; abortion; and in larger doses, shock and death. Accordingly, there are no currently available vaccines which induce antibody responses to LOS epitopes.

As shown in FIG. 1, the Lipid A portion of endotoxin generally comprises a hydrophilic backbone of glucosamine disaccharide which is either monophosphorylated or diphosphorylated (positions 1 and 4'); and which carries at least six molecules of ester-and amide-bound fatty acids. Four molecules of (R)-3-hydroxytetradecanoate (e.g. 3-hydroxy-myristoyl or β-hydroxymyristic acid or β-OH) are linked directly to the Lipid A backbone at positions 2, 3, 2', and 3'. Hydroxyl groups of two of the four molecules of β-OH are substituted with normal fatty acids (termed "secondary acyl chains", and including dodecanoate, tetradecanoate, and hexadecanoate) in forming acyloxyacyl groups.

One approach to making a detoxified endotoxin molecule involves isolating the endotoxin, and enzymatically-treating the isolated endotoxin with a human neutrophilic acyloxyacyl hydrolase (U.S. Patent Nos. 4,929,604, 5,013,661 and 5,200,184). The acyloxyacyl hydrolase hydrolyzes the fatty acids (nonhydroxylated, secondary acyl chains) from their ester linkages to hydroxy groups of O-OH (hydroxylated). The resultant altered endotoxin, from enzymatic treatment, contained a Lipid A moiety lacking non-hydroxylated fatty acids. This altered endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity. Another approach involves a method of modifying isolated

endotoxin by selectively removing the β-OH that is ester-linked to the reducing-end glucosamine backbone at position 3 (U.S. Patent No. 4,912,094; Reexamination Bl 4,912,094). The selective removal of O-OH was accomplished using alkaline hydrolysis. The resultant modified endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity. Both approaches involve chemically treating isolated endotoxin. Neither approach discloses the production in a gram negative bacterial pathogen of an endotoxin having substantially reduced toxicity, yet retaining antigenicity. Further, there has been no disclosure of the use of gram-negative bacteria, which have been engineered to produce an endotoxin having substantially reduced toxicity and yet retaining antigenicity, in a prophylactic or therapeutic vaccine against endotoxic shock and gram-negative bacteremia.

Summary of the Invention

The present invention is directed to a method for producing, in a mutant gram-negative bacterial pathogen, LOS which exhibits substantially reduced toxicity as compared to the wild type endotoxin, and which retains the antigenicity of its corresponding wild type endotoxin. The method comprises creating a mutation in a gene encoding a Lipid A fatty acid transferase (LAft) of the gram-negative bacterial pathogen, wherein the gene has been termed "laft" and the mutated gram-negative pathogen is termed "laft mutant." The mutation in the laft gene is such that there is a lack of functional LAft protein in the mutated gram-negative bacterial pathogen. It was found that Lipid A produced by the laft mutant lacks at least one fatty acid chains ("non-hydroxylated or secondary acyl chains", also known as "myristic acid moieties"). Thus, endotoxin isolated from the laft mutant exhibits substantially reduced toxicity and yet retains antigenicity, as compared to wild type. Endotoxin isolated from a laft mutant (either alone or conjugated with a carrier protein), or the laft mutant bacteria (whole cell vaccine), can be used to immunize individuals at risk of gram-negative bacteremia by inducing antibodies to the major antigenic determinants which reside in the carbohydrate structure of the complex carbohydrate structure of LOS. Further, the laft mutants can be engineered to

express heterologous antigens of other microbial pathogens at the surface of the mutated bacteria for presentation to a vaccinated individuals immune system in a multivalent vaccine. Also, the endotoxin isolated from the laft mutants of the present invention, either alone or conjugated with a carrier protein, may be used to generate LOS-specific antibody which may be useful for passive immunization and as reagents for diagnostic assays directed to detecting the presence of gram-negative bacterial pathogens in clinical specimens.

Brief Description of the Figures

FIG. 1 is a schematic representation of the general structure of lipid A of gram negative bacteria of the family *Enterobacteriaceae.*

Detailed Description of the Invention

"Endotoxin" is a term used herein for purposes of the specification and claims to refer to LOS of gram-negative bacterial pathogens, wherein the endotoxin is either in a cellassociated or isolated form. "laft endotoxin" refers to endotoxin isolated and purified from a gram-negative bacterial pathogen laft mutant.

"Vaccine candidate or vaccine antigen" is a term used herein for purposes of the specification and claims to refer to an endotoxin epitope having one or more of the following properties (a-d): (a) is immunogenic; (b) is surface-exposed (which can be shown by techniques known in the art including immunofluorescence assays, electron microscopy staining procedures, and by bactericidal assays); (c) induces antibody having bactericidal activity in the presence of complement and/or functions in immune clearance mechanisms; (d) induces antibody which neutralizes other functional activity of the epitope (immunogenicity, or toxicity, etc.).

"Gram-negative bacterial pathogen" is a term used herein for the purposes of the specification and claims to refer to one or more pathogenic (to humans or animals) bacterium of a genus and species including *Neisseria meningitides, Neisseria gonorrhoeae, Haemophilus influenzae, Haemophilus ducreyi, other Haemophilus* species, and *Moraxella catarrhalis).*

"Substantially reduced in toxicity" is a term used herein for the purposes of the specification and claims to refer to a reduction in bioactivity of at least 7 fold to 100 fold or more as compared to wild type endotoxin (either in isolated form or as part of the whole organism).

"Carrier protein" is a term used herein for the purposes of the specification and claims to refer to a protein which is conjugated to the laft endotoxin. While these endotoxins appears to be immunogenic on its own, it is known in the art that conjugation to a carrier protein can facilitate immunogenicity. Proteins which may be utilized according to the invention include any protein which is safe for administration to mammals and which may serve as an immunologically-effective carrier protein. In particular embodiments, cell surface proteins, membrane proteins, toxins and toxoids may be used. Criteria for choice of carrier protein would include absence of primary toxicity, minimal risk of allergic reaction, and the age and immune status of the individual. With these criteria in mind, carrier proteins known to those skilled in the art may include, but are not limited to, *Salmonella flagellin, Haemophilus pilin, Pseudomonas pili, Pseudomonas* exotoxin, outer membrane proteins of *Haemophilus* (51 kDa, 28-30 kDa, and 40 kDa membrane proteins) or *N. meningitides* or *N. gonorrheae, Escherichia coli* heat labile enterotoxin LTB, cholera toxin, pneumolysin of *S. pneumoniae,* viral proteins including rotavirus VP7 and respiratory syncytial virus F and G proteins, diphtheria toxoids, tetanus toxoids, and diphtheria toxin cross-reactive mutant protein ("CRM"). There are several methods known in the art for conjugating endotoxin to a carrier protein. Such methods may include, but are not limited to, the use of glutaraldehyde, or succinimidyl m-maleimidobenzoate, or 1ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, or by using bromoacetylated carrier protein (see, e.g. Robey et al., 1989, *Anal. Biochem.* 177:373-377). Conjugation of endotoxin to carrier proteins can be performed by a variety of methods such as by direct conjugation to the carrier protein by cyanogen bromide, reductive amination, or by using bifunctional linkers. Such bifunctional linkers include, but are not

limited to, N-hydroxy succinimide-based linkers cystamine, glutaraldehyde, and diamino hexane.

"Laft gene" is a term used herein for the purposes of the specification and claims to refer to a gene in a gram-negative bacterial pathogen (as defined herein) which has a nucleotide sequence the same or substantially similar (80% or more identity) to SEQ ID NO:1; and has limited identity and homology to the *msbB* gene of *E. coli.* It was discovered in the development of the present invention that the laft gene has a functional role in the biosynthesis, and hence structure, of Lipid A. A "laft mutant" is a term used herein for the purposes of the specification and claims to refer to a mutation in the laft gene of a gram-negative bacterial pathogen (as defined herein). The methods and compositions of the present invention relate to LOS biosynthetic pathways of gram-negative bacterial pathogens. More specifically, the present invention relates to identification of a gene (laft) involved in the transfer of fatty acids to the Lipid A portion during LOS biosynthesis. A mutation of the laft gene in gram-negative bacterial pathogens results in mutant bacteria bearing endotoxin which is substantially reduced in toxicity, and yet retains antigenicity, as compared to wild type bacteria of the same species. The genetics of Lipid A biosynthesis of enteric bacteria, as it was known at the time of the present invention, is summarized in Schnaitman and Klena (1993, *Microbiol. Rev.* 57:655-682). Genes *lpxA*, *lpxB*, *lpxC*, and *lpxD* encode gene products which function on the glucosamine backbone of Lipid A including transfer of β-hydroxymyristic acid to glucosamine. The htrB gene of *E. coli* was described as a gene that affects the inner core structure (KDO, heptose, phosphorylethanolamine (PEA)) which was discovered during a screen for genes necessary for growth of *E. coli* at elevated temperatures. Knockout mutations of htrb resulted in mutant *E. coli* which exhibited a reduced sensitivity to deoxycholate, an inability to grow at temperatures above 32.5°C, and a decrease in LPS staining intensity (Schnaitman et al., 1993, *supra;* Karow et al., 1992, J. Bacteriol. 174:7407-7418). Karow et al. further noted that at between about 30°C to about 42°C, *E.coli htrb* mutants have changes in the fatty acid

composition of both LPS and phospholipids, and particularly, overproduce phospholipids, as compared to wild type.

The *msbB* gene of E.coli was described as a multicopy suppressor of the htrB gene (Karow and Georgopoulos, 1992, *J.* Bacteriol. 174:702-710). The protein encoded by the *E. coli msbB* gene has an amino acid sequence similar to that of the HtrB protein of *E. coli.* An *E. coli htrB-msbB* double mutant demonstrated different morphological characteristics at 30°C as compared to that displayed by either an *E. coli htrB* mutant or the wild type (Karow and Georgopoulos, 1992, supra). Additional studies with an *E. coli msbB* mutant showed that (a) the LPS lacks the myristoyl fatty acid moiety of the Lipid A portion, and (b) that LPS isolated from the *E. coli msbB* mutant had a 1,000-10,000 fold reduction in the ability to stimulate TNFA production from adherent monocytes and E-selectin production by human endothelial cells (Somerville et al., 1996, *J. Clin. Invest.* 97:359-365).

The discoveries comprising the present invention include the finding of a novel gene for LOS bisoynthesis- "laft" gene, and the discovery that knockout mutations of the laft gene of gram-negative bacteria result in laft mutants which specifically lack one or more secondary acyl chain fatty acids which are ester-bound to the hydroxyl groups of two of the four molecules of β-OH. Thus, it appears that the LAft protein has either acyltransferase activity, or indirectly or directly affects regulation of acyltransferase activity.

The following examples are presented to illustrate preferred embodiments of aspects of the present invention, and are not intended to limit the scope of the invention. In particular, a preferred embodiment is the making of an *H. influenzae* laft mutant, and methods of using the same as a whole cell, or to isolate therefrom the endotoxin, in vaccine preparations or to generate antibodies for therapeutic or diagnostic applications. However, since the Lipid A moiety of LOS is highly conserved among bacteria containing LOS characterized to date, the invention relates to gram-negative bacterial pathogens, as defined previously herein. There may be microheterogeneity in terms of the length of the secondary acyl chain (12 or 14 carbon chains) and to which of the

four β-OH are substituted (1, 2, or 4) (Erwin et al., 1991, *Infect Immun* 59:1881-1887); however, the nature of the substitution is the same and thus the particular steps (genes and gene products) involved in the biosynthetic pathway appear conserved. For example, removal of secondary acyl chains from various gram-negative bacterial pathogens (e.g., *H. influenzae* and N. *meningitides)* using human acyloxyacyl hydrolase resulted in deacylated LOS from all species tested having significantly reduced mitogenic activity (Erwin et al., 1991, *supra) as* compared to the respective wild type strain.

EXAMPLE 1

Generation of laft mutants

One approach to identifying a gene involved in Lipid A synthesis of LOS was to search an *H. influenzae* genome database for a gene having some homology with the *E. coli msbB* gene. Additionally, the database was searched for an open reading frame which contained a gene resembling that of the *E. coli msbB* gene. Each search resulted in the identification of the same open reading frame, "HI1099". The sequence of this open reading frame is shown as SEQ ID NO:l. Based on SEQ ID NO:l, two internal oligonucleotide primers were synthesized (SEQ ID NOs: 2 and 3). These primers were then used to amplify this gene from H. influenzae strain 2019 DNA using polymerase chain reaction under standard conditions.

The resultant fragment of approximately 800 bp was cloned into a plasmid vector (PCR2.1) in forming a recombinant plasmid termed pLAft. The 800 bp fragment was used as a probe to screen a genomic library of *H. influenzae* strain 2019 (constructed in plasmids) . A plasmid, PD,IV, was identified by hybridization to this probe, and then isolated. PD,IV contained an insert of approximately 1.7 kb. Sequence analysis of this insert confirmed that the insert contained in this plasmid comprised the *H. influenzae* laft gene, which was essentially identical in sequence to the open reading frame of HI1099 (SEQ ID NO:l).

This insert was cloned into an H. influenzae-compatible plasmid (pACYC184), and also a kanamycin resistance cassette was cloned into a unique

*Sph*I restriction enzyme site in the laft gene of the insert. The resultant plasmid, containing the H. *influenzae* laft gene, was restricted with restriction enzyme EcoRI, and transformed into *H. influenzae* strain 2019. The resultant transformants were grown at 30°C on growth media containing lg/ml ribistomycin. Colonies were screened by hybridization with the PMSBB fragment, and genomic DNA was made from clones that tested positive by hybridization. Southern blot analysis of the genomic DNA demonstrated that the kanamycin resistant cassette was introduced by recombination into the genomic DNA and into the laft gene. Analysis of the LOS by SDSPAGE of one of these transformed clones of *H. influenzae* strain 2019 showed a single band of LOS which migrated slightly faster that that of the wild type *H. influenzae* strain 2019, indicating that a mutation had occurred in the laft gene which then affected LOS structure. This *H. influenzae* laft mutant strain was designated BK-1.

It will be appreciated by those skilled in the art, that there are various standard techniques known to those skilled in the art for mutating a bacterial gene such as the laft gene. Thus, the identification of the laft gene of H. influenzae as taught in this specification enables one skilled in the art to mutate the laft gene of an *H. influenzae* strain by any one of those techniques, including site-directed mutagenesis, and shuttle mutagenesis using transposons. For example, transposon mutagenesis was used successfully to generate htrB mutants of H. *influenzae,* as described in more detail (U.S. patent application Serial No. 08/565,943, herein incorporated by reference, and assigned to the assignee of the present invention). Such techniques can be applied by those skilled in the art to generate laft mutants.

EXAMPLE 2

Characterization of Endotoxin of laft mutants

LOS from BK-1, the *H. influenzae* laft mutant, was isolated using the phenol-chloroform-ether method. The BK-1 LOS was first tested to determine whether the observed change in the LOS migration pattern by SDS-PAGE of BK-1 was due to a change in the oligosaccharide portion of the LOS caused by mutating the laft gene. In an initial analysis, the BK-1 LOS was tested in an

immunodot assay for immunoreactivity with monoclonal antibody 6E4 (MAb 6E4). MAb 6E4 recognizes an epitope (2-keto-3-deoxyoctulosonic acid) on the oligosaccharide portion of LOS, and has been used in determining LOS phenotypes of mutant Neisseria (Stephens et al., 1994, *Infect. Immun.* 62:2947-52) and mutant *Haemophilus* (McLaughlin et al., 1992, *J. Bacteriol.* 174:6455-9). BK-1 LOS was strongly immunoreactive with MAb 6E4 indicating that the change in LOS structure, as evidenced by its change in SDSPAGE migration pattern, is not related to changes in the oligosaccharide portion of the LOS.

Mass spectroscopy analysis confirmed that the oligosaccharide structure of BK-1 LOS was essentially unchanged from that found in *H. influenzae* strain 2019 (wild type). Using methods similar to those disclosed for analysis of htrB mutants (see, e.g., U.S. patent application Serial No. 08/565,943, herein incorporated by reference), the Lipid A portion of BK-1 LOS was also analyzed by mass spectroscopy. Briefly, the *H. influence* laft mutant and wild type LOS were each analyzed by electrospray ionization-mass spectrometry (ESI-MS) to provide molecular mass profiles for the different components of LOS. LOS was first isolated from the respective strains using any one of the several methods known in the art (e.g., phenol-water method, Westphal et al., 1965, *Methods in Carbohydrate Chemistry* 5:83-91; proteolytic digestion, Hitchcock et al., 1983, *LT. Bacteriol.* 154:269-277). The isolated LOS species were then 0-deacylated by mild hydrazine treatment (37°C for 20 minutes; see Phillips et al., 1990, *Biomed. Environ. Mass Spectrom.* 19:731-745), and subjected to analysis by ESI-MS. The results confirm that the change in the LOS structure of BK-1 is due to changes in the secondary acyl chains as compared to the Lipid A of *H. influenzae* strain 2019 (wild type).

EXAMPLE 3

Substantially reduced toxicity of laft mutants

The effect due to the lack of one or more secondary acyl chains on the toxicity of a gram-negative bacterial pathogen was examined using a standard *in vitro* assay for measuring *in vivo* toxicity. Using similar methods as described in more detail for htrB mutants (U.S. patent application Serial No. 08/565,943;

herein incorporated by reference), BK-1 LOS was compared to LOS isolated from *H. influenzae* strain 2019 in their ability to stimulate secretion of TNFα. The amount of TNFα, directly proportional to the toxicity of the stimulating LOS, was measured in a TNFα ELISA (available commercially from Biosource) using cell-free supernatant from human macrophage cell line THP1 exposed to the LOS to be evaluated. Briefly, the amount of TNFα can be measured by (a) removing the cell-free supernatant containing the TNFα; (b) adding the supernatant to a TNFα-sensitive cell line, such as WEHI 164; and (c) measuring the resultant cytotoxicity (See for example, Espevik et al., 1986, *J Immunol Methods* 95:99; Sakurai et al., 1985, *Cancer Immunol Immunother* 20:6-10; Adams et al., 1990, *J Clin Microbiol* 28:998-1001; Adams et al., 1990, *J Leukoc Biol* 48:549-56; Tsai et al., 1992, *Cell Immunol* 144:203-16; and Pfister et al., 1992, *Immunol* 77:473-6).

Using this assay, 100 ng of *H. influenzae* strain 2019 LOS induced the release of an average of 313 pg of TNFα, as compared to the release of 1 pg of TNFα induced by BK-1 LOS. Thus, the LOS from the BK-1 laft mutant shows a statistically significant reduction (approximately 300 fold; p-value of less than 0.0001) in its ability to stimulate TNFα release. This reduced ability to stimulate TNFα is one indication of the laft mutant being substantially reduced in toxicity due to the lack of one or more secondary acyl chains in the Lipid A portion of the endotoxin.

An additional assay was performed, as another indicia of the substantial reduction in toxicity exhibited by BK-1 LOS. The limulus amoebocyte lysate assay ("LAL") is a standard test known by those skilled in the art for evaluating the toxicity of LOS (Mertsola et al., 1990, *J. Clin. Microbiol.* 28:2700-06). Using this standard assay *H. influenzae* strain 2019 LOS gave a response of 12.5 EU/ng (EU is endotoxin units), as compared to a response between 0.125 EU/ng to 0.0125 EU/ng by BK-1 LOS. Thus, the LOS from the BK-1 laft mutant shows a statistically significant reduction (approximately 100 to 1000 fold) in endotoxin activity. This reduced activity is an indication, in addition to that from the TNFα assay, that the laft mutant is substantially reduced in toxicity due

to the lack of one or more secondary acyl chains in the Lipid A portion of the endotoxin.

EXAMPLE 4

Use of laft mutants as immunogens

In one aspect of this embodiment, the laft mutant gramnegative bacteria is used as a whole cell vaccine. The benefit of using live, attenuated (weakened in its ability to cause pathogenesis) bacteria as an immunogen in a vaccine formula is that they are able to survive and may persist in the human or animal body, and thus confer prolonged immunity against disease. In conjunction with the benefit of using a live bacteria to prolong the immune response, laft mutants have the added benefit in that they exhibit substantially reduced toxicity. Another advantage, as compared to a vaccine formulation comprising an isolated peptide representing a bacterial antigen, is that a bacterial antigen expressed on the surface of a bacterial cell will often result in greater stimulation of the immune response. This is because the surface of bacteria of the family *Enterobacteriaceae* acts as a natural adjuvant to enhance the immune response to an antigen presented thereon (Wezler, 1994, *Ann NY Acad Sci* 730:367-370). Thus, using a live bacterial vaccine, such as a laft mutant, to express complete proteins in a native conformation (i.e., as part of the bacterial outer membrane) is likely to elicit more of a protective immune response than an isolated protein alone.

Live bacterial vaccine vectors of the family *Enterobacteriaceae* that have been described previously include attenuated *Salmonella* strains (Stocker et al., U.S. Patent Nos. 5,210,035; 4,837,151; and 4,735,801; and Curtiss et al., 1988, Vaccine 6:155-160; herein incorporated by reference), and *Shigella flexneri* (Sizemore etl al., 1995, *Science* 270:299-302; herein incorporated by reference). One preferred embodiment is to provide a vaccine delivery system for human or animal (depending on the genus and species of the gram-negative bacterial pathogen) mucosal pathogens. Thus, immunization by the parental route or by the mucosal route with an immunologically effective amount of the laft mutant, or a laft mutant transformed to recombinantly express additional bacterial

antigens (that do not negatively affect the growth or replication of the transformed laft mutant), can lead to colonization of mucosal surfaces to induce mucosal immunity against the antigens displayed on the surface of, or secreted from the LOS mutant. The resultant laft mutant can be used in a vaccine formulation which expresses the bacterial antigen(s). Similar methods can be used to construct an inactivated laft mutant vaccine formulation except that the laft mutant is inactivated, such as by chemical means known in the art, prior to use as an immunogen and without substantially affecting the immunogenicity of the expressed immunogen(s). For example, human bronchial mucosal immunity has been stimulated with an aerosol vaccine comprising lysed *H. influenzae* (Latil et al., 1986, *J Clin Microbiol* 23:1015-1021). Either of the live laft mutant vaccine or the inactivated laft mutant vaccine may also be formulated with a suitable adjuvant in order to further enhance the immunological response to the antigen(s) expressed by the vaccine vector, as to be described in more detail.

In another aspect of this embodiment, the laft endotoxin is isolated from the laft mutant using methods known in the art, and the isolated endotoxin is used in a vaccine formulation. As mentioned previously, the endotoxin may be conjugated to a carrier protein in forming an endotoxin conjugate which can be used as the immunogen, in an immunologically effective amount, in the vaccine formulation. The advantage of using endotoxin isolated from a laft mutant of a gram-negative bacterial pathogen is that it lacks one or more secondary acyl chains, and thus exhibits substantially reduced toxicity as compared to endotoxin isolated from the respective wild type bacteria. Therefore, endotoxin isolated from a laft mutant of a gram-negative bacterial pathogen can be used by itself, or in an endotoxin conjugate, in a vaccine formulation in inducing immunity against the respective wild type gram-negative bacterial pathogen.

Many methods are known for the introduction of a vaccine formulation into the human or animal (collectively referred to as "individual") to be vaccinated. These include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal, and oral

administration. Conventionally, vaccine formulations containing either live bacteria, or attenuated or inactivated bacteria, are administered by injection or by oral administration. For example, respiratory immunity can be stimulated by intestinal immunization with purified *H. influenzae* antigens (Cripps et al., 1992, *J. Infect Dis* 165Sl:Sl99-201; herein incorporated by reference). The vaccine formulation may comprise a pharmaceutically acceptable carrier as a medium in which the laft mutant bacterial cells or LOS isolated from the laft mutant (in isolated or conjugate form) is included. Various adjuvants may be used in conjunction with vaccine formulations. The adjuvants aid by modulating the immune response and in attaining a more durable and higher level of immunity using smaller amounts of vaccine antigen or fewer doses than if the vaccine antigen were administered alone. Examples of adjuvants include incomplete Freund's adjuvant, Adjuvant 65 (containing peanut oil, mannide, monooleate and aluminum monostearate), oil emulsions, Ribi adjuvant, the pluronic polyols, polyamines, Avridine, Quil A, saponin, MPL, QS-21, and mineral gels such as aluminum hydroxide, aluminum phosphate, etc. The vaccine formulation is administered in a prophylactically effective amount to be immunogenic, which depends on factors including the individuals ability to mount an immune response, the degree of protection to be induced, and the route of administration.

In another aspect of the invention, the vaccine formulation can be administered orally by including it as part of the feed given to economically important livestock. As known by those skilled in the art, and for example, species of *Haemophilus* and *Moraxella* are pathogenic for economically important livestock. Using the methods according to the present invention, as illustrated in the following examples, laft mutants of such animal pathogens can be produced. The resultant laft mutants, or endotoxin isolated therefrom (in isolated or conjugate form), can be used in a vaccine formulation. Use of vaccine formulations, containing one or more antigens of various microbial pathogens, in animal feed has been described previously (See for example, Pritchard et al., 1978, *Avian Dis* 22:562-575).

EXAMPLE 5

Neisseria mutants as immunogens

N.gonorrhoeae is a gram-negative bacterial pathogen causing the sexually transmitted disease gonorrhea, which subsequently can lead to pelvic inflammatory disease in females. *N. meningitides* is a gram-negative bacterial pathogen which can cause a variety of clinical infections including bacteremia, septicemia, meningitis, and pneumonia. In another embodiment, using the methods according to the present invention, a laft mutant from a strain of *Neisseria* selected from the group including *Neisseria qonorrhoeae, and Neisseria meningitidis,* can be produced and identified. The resultant *Neisseria laft* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, endotoxin isolated from a *Neisseria laft* mutant can be used (by itself or in conjugated form) in a vaccine formulation in inducing immunity against the wild type strains of *Neisseria* pathogens. Such LOS (by itself or in conjugated form) may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), and an adjuvant.

Alternatively, *Neisseria laft* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Neisserial* species are known to those skilled in the art, including: **pLES2** confers ampicillin resistance, is a shuttle vector functional in both *E. coli* and *N. gronorrhoeae,* and contains a polylinker with restriction sites for EcoRI, *Smal, and* BaffEI (Stein et al., 1983, Gene 25:241-247).

Neisseria species also contain a natural transformation process (Rudel et al., 1995, *Proc Natl Acad Sci USA* 92:7896-90; Goodman et al., 1991, *J*

Bacteriol 173:5921-5923); and can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 6

Haemophilus mutants as immunogens

H.ducreyi is a gram-negative bacterial pathogen causing a genital ulcer disease, chancroid. Other *Haemophilus* species (e.g. *suis, somnus, parahemolyticus)* are important animal pathogens. Using the methods according to the present invention, *Haemophilus* laft mutants can be produced and identified. The resultant *Haemophilus laft* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, endotoxin isolated from a *Haemaphilus laft* mutant can be used (by itself or in conjugated form) in a vaccine formulation in inducing immunity against the respective wild type strains of *Haemophilus.* Such LOS (by itself or in conjugated form) may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), and an adjuvant.

Alternatively, *Haemophilus laft* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Haemophilus* species are known to those skilled in the art, as previously illustrated herein (see also U.S. Serial No. 08/565,943).

EXAMPLE 7

Moraxella catarrhalis laft mutants as immunogens

Moraxella catarrhalis is a gram-negative bacterial pathogen causing otitis media in children; sinusitis and conjunctivitis in children and adults; and lower respiratory tract infections, septicemia, and meningitis in immunocompromised hosts. In another embodiment of the present invention, M.

catarrhalis laft mutants can be produced and identified using the methods according to the present invention. The resultant M. *catarrhalis laft* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, endotoxin isolated from a *M. catarrhalis laft* mutant can be used (by itself or in conjugated form) in a vaccine formulation in inducing immunity against the wild type strains of *M. catarrhalis.* The endotoxin isolated from the *M. catarrhalis laft* mutant may be used (by itself or in conjugated form) in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), and an adjuvant.

Alternatively, *M. catarrhalis laft* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *M. catarrhalis* are known to those skilled in the art. *M. catarrhalis* contains a natural transformation process (Juni, 1977, *J Clin Microbiol* 5:227-35) and can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 8

Multivalent laft mutant vaccine formulation

In one embodiment according to the present invention, the laft mutant is genetically engineered to express one or more heterologous microbial antigens in producing a multivalent vaccine using methods known to those skilled in the art. In a preferred embodiment, a microbial pathogen may include a respiratory pathogen selected from the group of pathogens, with respective antigens, in Table 1.

Table 1

PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
H. influenzae	otitis media, lower respiratory tract	D-15, P1, P6[1]
Group A Streptococcus	pharyngitis, rheumatic fever	M[2]
Branhamella catarrhalis	otitis media, lower respiratory tract	CD, E[3]
Streptococcus pneumoniae	pneumonia, otitis media, meningitis	autolysin, pneumolysin[4]
Bordetella pertussis	pertussis (whooping cough)	filamentous hem-agglutinin, pertussis toxin, 69kDa Omp[5]
Pseudomonas aeruginosa	respiratory tract	Omp OprF, exotoxin A[6]
Legionella pneumophila	pneumonia	OmpS, Hsp60[7]
Mycoplasma pneumoniae	upper and lower respiratory tract	P1[8]
Respiratory syncytial virus	lower respiratory tract	M2, P, F, G[9]
Influenza virus	influenza	HA, M[10]
Adenovirus	common cold	
rhinovirus	common cold	VP1, VP2, VP3[11]
Parainfluenza virus	common cold	HN, F[12]
Pneumocystis carinii	pneumonia in AIDS	msg[13]

[1]- (Flack et al., 1995 *Gene* 156:97-99; Panezutti et al., 1993, 61:1867-1872; Nelson et al., 1988, *Rev Infect Diseases* 10:S331-336).
[2]- (Pruksakorn et al., 1994, *Lancet* 344:639-642; Dole et al., 1993, *J Immunol* 151:2188-94).
[3]- (Murphy et al., 1989, *Infect Immun* 57:2938-2941; Faden et al., 1992, *Infect Immun* 60:3824-3829).
[4]- (Lock et al., 1992, *Microb Pathog* 12:137-143).
[5]- (Novotny et al., 1991, *Dev Biol Stand* 73:243-249; Lipscombe et al., 1991, *Mol Microbiol* 5:1385-1392; He et al., 1993, *Eur J Clin Microbiol Infect Dis* 12:690-695).
[6]- (Rawling et al., 1995, *Infect Immun* 63:38-42; Pennington et al., 1988, *J Hosp Infect* 11A:96-102).
[7]- (Weeratna et al., 1994, *Infect Immun* 62:3454-3462).

8-(Jacobs et al., 1990, *Infect Immun* 58:2464-2469; 1990, *J Clin Microbiol* 28:1194-1197).

9- (Kulkarni et al., 1995, *J Virol* 69:1261-1264; Leonov et al., 1994, *J Gen Virol* 75:1353-1359; Garcia et al., 1993, *Virology* 195:239-242; Vaux-Peretz et al., 1992, *Vaccine* 10:113-118).

10- (Kaly et al., 1994, Vaccine 12:753-760; Bucher et al., 1980, *J* Virol 36:586-590).

11- (Francis et al., 1987, *J Gen Virol* 68:2687-2691).

12- (Morein et al., 1983, *J Gen Virol* 64:1557-1569).

13- (Garbe et al., 1994, *Infect Immun* 62:3092-3101).

In another preferred embodiment, a microbial pathogen may include a pathogen causing a sexually transmitted disease selected from the group of pathogens, with respective antigens, in Table 2.

Table 2

PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
N. gonorrhoeae	gonorrhea	IgA1 protease[1], PIB[2], H.8[3], Por[4]
Chlamydia trachomatis	nongonococcal urethritis	MOMP[5], HSP[6]

1- (Lomholt et al., 1994, *Infect Immun* 62:3178-83).

2_ (Heckels et al., 1990, *Vaccine* 8:225-230).

3_ (Blacker et al., 1985, *J Infect Dis* 151:650-657).

4_ (Wetzler et al., 1992, *Vaccine* 8:225-230).

5_ (Campos et al., *1995, Ophthamol Vis Sci* 36:1477-91; Murdin et al., 1995, *Infect Immun* 63:1116-21).

6_ (Taylor et al., 1990, *Infect Immun 58:3061-3).*

Tables 1 & 2, and the references footnoted which are herein incorporated by reference, illustrate various protein antigens, or peptides thereof, viewed by those skilled in the art to be useful as vaccine candidates against the respective microbial pathogen. Typically, the immunopotency of an epitope, whether from a protein or peptide, of a microbial pathogen is determined by monitoring the immune response of an animal following immunization with the epitope and/or by analyzing human convalescent sera in conjunction with pre-immune sera. Thus, one skilled in the art can determine protein or peptide antigens from microbial pathogens which would be desired to include as a heterologous antigen to be expressed by an LOS mutant according to the present invention. A corresponding nucleic acid sequence, the encoding sequence, can then be deduced from the amino acid sequence of the protein or peptide antigen, wherein the encoding sequence is introduced into the LOS mutant for expression.

EXAMPLE 9

Use of laft mutants to generate antisera

The laft mutant, or endotoxin purified therefrom (by itself or in conjugated form), can be used to generate endotoxinspecific antisera, directed to the particular gram-negative bacterial pathogen, which can be used in an immunoassay to detect the antigen (that particular gram-negative bacterial pathogen), present in the body fluid of an individual suspected of having an infection caused by that gram-negative bacterial pathogen. The body fluid(s) collected for analysis depend on the microorganism to be detected, the suspected site of infection, and whether the body fluid is suspected of containing the antigen or containing antisera. With those considerations in mind, the body fluid could include one or more of sputum, blood, cerebrospinal fluid, lesion exudate, swabbed material from the suspected infection site, and fluids from the upper respiratory tract. Inununoassays for such detection comprises any immunoassay known in the art including, but not limited to, radioimmunoassay, ELISA,"sandwich" assay, precipitin reaction, agglutination assay, fluorescent immunoassay, and chemiluminescence-based immunoassay.

Alternatively, where an inununocompromised individual is suffering from a potentially life-threatening infection caused by a particular gram-negative bacterial pathogen, immunization may be passive, i.e. immunization comprising administration of purified human immunoglobulin containing antibody against a laft mutant or endotoxin isolated therefrom.

It should be understood that while the invention has been described in detail herein, the examples were for illustrative purposes only. Other modifications of the embodiments of the present invention that are obvious to those skilled in the art of molecular biology, medical diagnostics, and related disciplines are intended to be within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Iowa Research Foundation et al.

(ii) TITLE OF INVENTION: LAFT MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Unknown
(B) FILING DATE: 28-MAY-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Viksnins, Ann S
(B) REGISTRATION NUMBER: 37,748
(C) REFERENCE/DOCKET NUMBER: 875.003WO1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612-373-6961
(B) TELEFAX: 912-339-3061
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 954 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGTCGGATA ATCAACAAAA TTTACGTTTG ACGGCGAGAG TGGGCTATGA AGCGCACTTT 60
TCATGGTCGT ATTTAAAGCC TCAATATTGG GGGATTTGGC TTGGTATTTT CTTTTTATTG 120
TTGTTAGCAT TTGTGCCTTT TCGTCTGCGC GATAAATTGA CGGGAAAATT AGGTATTTGG 180
ATTGGGCATA AAGCAAAGAA ACAGCGTACG CGTGCACAAA CTAACTTGCA ATATTGTTTC 240
CCTCATTGGA CTGAACAACA ACGTGAGCAA GTGATTGATA AAATGTTTGC GGTTGTCGCT 300
CAGGTTATGT TTGGTATTGG TGAGATTGCC ATCCGTTCAA AGAAACATTT GCAAAAACGC 360
AGCGAATTTA TCGGTCTTGA ACATATCGAA CAGGCAAAAG CTGAAGGAAA GAATATTATT 420
CTTATGGTGC CACATGGCTG GGCGATTGAT GCGTCTGGCA TTATTTTGCA CACTCAAGGC 480
ATGCCAATGA CTTCTATGTA TAATCCACAC CGTAATCCAT TGGTGGATTG GCTTTGGACG 540
ATTACACGCC AACGTTTCGG CGGAAAAATG CATGCACGCC AAAATGGTAT TAAACCTTTT 600
TTAAGTCATG TTCGTAAAGG CGAAATGGGT TATTACTTAC CCGATGAAGA TTTTGGGGCG 660
GAACAAAGCG TATTTGTTGA TTTCTTTGGG ACTTATAAAG CGACATTACC AGGGTTAAAT 720
AAAATGGCAA AACTTTCTAA AGCCGTTGTT ATTCCAATGT TTCCTCGTTA TAACGCTGAA 780
ACGGGCAAAT ATGAAATGGA AATTCATCCT GCAATGAATT TAAGTGATGA TCCTGAACAA 840
TCAGCCCGAG CAATGAACGA AGAAATAGAA TCTTTTGTTA CGCCAGCGCC AGAGCAATAT 900
GTTTGGATTT TGCAATTATT GCGTACAAGG AAAGATGGCG AAGATCTTTA TGAT 954

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAAGCGCACT TTTCATGG 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTTCGCCATC TTTCCTTG 18

WHAT IS CLAIMED IS:

1. A bacterium having a mutated Lipid A fatty transferase gene wherein the mutation results in a lack of Lipid A fatty acid transferase activity in the bacterium.

2. The bacterium of claim 1 which is a gram negative bacterium.

3. Endotoxin isolated from the bacterium of claim 1.

4. A method of preparing mutant endotoxin comprising isolating endotoxin from the bacterium of claim 1. Wherein the isolated endotoxin has substantially reduced toxicity relative to the endotoxin from the corresponding wild type bacterium.

5. A method of immunizing an individual to prevent a prevent caused by a gram negative bacterial pathogen comprising administering to the individual an effective amount of the bacterium of claim 1.

6. The method of claim 5 wherein the bacterium is live.

7. The method of claim 5 wherein the bacterium is inactivated prior to administration.

8. The method of claim 5 wherein the individual is human.

9. The method of claim 5 wherein the administration is intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal or oral.

10. A vaccine formulation comprising an effective amount of bacteria of claim 1.

11. A vaccine formulation of claim 10 wherein the bacterium is live.

12. The vaccine formulation of claim 10 wherein the bacterium is inactivated.

13. The vaccine formulation of claim 10 further comprising a physiological carrier and an adjuvant.

14. The vaccine formulation of claim 10 further comprising a physiological carrier suitable for mucosal administration.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10881

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/02; A01N 63/00; C12N 1/00, 1/20

US CL :424/234.1, 236.1, 93.1, 93.2, 93.4, 93.48; 435/243, 252.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/234.1, 236.1, 93.1, 93.2, 93.4, 93.48; 435/243, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Please See Extra Sheet.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, TOXLINE, BIOSIS, EMBASE, CABA, AGRIS, PASCAL, JAPIO, JICST-EPLUS, EUROPAT, DERWENT, DISSERTATION ABSTRACTS, INSIDE CONFERENCES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE et al. Mutation of the htrB locus of Haemophilus influenzae nontypable strain 2019 is associated with modifications of lipid A and phosphorylation of the lipooligosaccharide. J. Biol. Chem. 10 November 1995, Vol. 270, No. 45, pages 27151-27159, especially pages 27157 and 27158.	1-4 ------ 5-14
X -- Y	SOMERVILLE et al. A novel Escherichia coli lipid A mutant that produces an antiinflammatory lipopolysaccharide. J. Clin. Invest. January 1996, Vol. 97, No. 2, pages 359-365, see entire document.	1-4 ------ 5-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 AUGUST 1998	24 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer S. DEVI aco Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10881

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	CLEMENTZ et al. Function of the Escherichia coli msbB gene, a multicopy suppressor of htrB knockouts, in the acylation of lipid A. J. Biol. Chem. 18 April 1997, Vol. 272, No. 16, pages 10353-10360, espeically pages 10353 and 10359.	1-4 ------ 5-14
Y, P	US 5,641,492 A (SPROUSE ET AL.) 24 June 1997 (06/24/97), see columns 2-4, 15 and 16.	1-14
X, P ------ Y, P	SUNSHINE et al. Mutation of the htrB gene in a virulent Salmonella typhimurium strain by intergeneric transduction: Strain construction and phenotypic characterization. J. Bacteriol. September 1997, Vol. 179, No. 17, pages 5521-5533, especially pages 5531 and 5532.	1-4 ------ 5-14
Y, P	JONES et al. Study of the role of the htrB gene in Salmonella typhimurium virulence. Infect. Immun. November 1997, Vol. 65, No. 11, pages 4778-4783, especially page 4781.	10 and 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10881

B. FIELDS SEARCHED
Documentation other than minimum documentation that are included in the fields searched:

Search terms: Gram negative bacterial mutants, mutated lipid A fatty transferase, LAFT, vaccine with lipid A mutants, non-toxic LPS/LOS, inventor name search